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The Phagocytes in Hemolymph of *Halocynthia roretzi* and Their Phagocytic Activity

Shin-Ichi Ohtake, Takeyuki Abe, Fumio Shishikura, and Kunio Tanaka

Department of Biology, Nihon University School of Medicine, Oyaguchi Itabashi, Tokyo 173, Japan

ABSTRACT—The phagocytes in hemolymph of *Halocynthia roretzi* and their phagocytic activity *in vitro* were studied by electron microscopy. Hemocytes were ultrastructurally classified as follows: Large granular amebocytes (LGs), small granular amebocytes (SGs), eight types of vacuolated or vesicular cells, dense granular cells and lymphoid cells. The vacuolated or vesicular cells, V1-V8, were distinguished from each other by the size of vacuole and the nature of inclusions. The identification of phagocytic cells and possible targets were examined *in vitro*. Freshly collected hemolymph was incubated with latex beads (LB) of 1, 5, 26 μm in diameter, SRBC, *Escherichia coli* or small pieces of tunic. Small granular amebocytes actively ingested all the particles except the 26 μm-LB. They surrounded the tunic pieces and the 26 μm-LB. LGs ingested only 1 μm-LB. Their activity was, however, weaker than that of SGs. We could not find any phagocytosed particles in other hemocytes during a 30 min incubation. Furthermore, the plasma factor(s) that activated the phagocytosis to SRBC, did not influence that to the LB.

INTRODUCTION

The basic strategies of self defense mechanism of higher vertebrates are the innate immunity including phagocytosis or encapsulation and the acquired immunity. Immunological specificity and acquired memory are essential features of vertebrate immunological competence [19]. Tunicates and vertebrates are thought to have derived from the same ancestor. However, evidence concerning immunological responses on tunicates is limited. As in vertebrates, the recognition and reaction system of foreignness is a function of the hemocytes and hemolymph plasma in ascidians. Humoral defense factors reported on ascidian hemolymph are natural aggulutinins [3, 12, 32, 34, 36] and antimicrobial factors [2, 20]. Immunoglobulins and complement cascade have, however, not yet been determined in the plasma of these animals. Immunological specificity and memory are yet to be clearly demonstrated in ascidians.

The innate cellular defense mechanisms such as phagocytosis to foreign materials may be important in these animals. Many authors observed phagocytosis or encapsulation when foreign bodies were introduced into the tunic, or the vascular system. For example, carbon particles which were injected into the tunic of the body wall of *Halocynthia aurantium* were phagocytosed by hyaline amebocytes [30], glass fragments which were inserted into the branchial sac of *Molgula manhattensis* were encapsulated by vanadocytes [1] and parasitic copepods in some ascidians were encapsulated by host cells [6, 23]. Recently, electron microscopic observations of hemocytes of ascidians have accumulated, although the knowledge of functional characterization of the cellular reaction to defense mechanism is still limited [4, 7, 15, 22, 24, 32, 37]. We therefore wanted to define the phagocytic cell of *H. roretzi*. This ascidia can be purchased from cultivators and kept in a laboratory aquarium, and the responses of its hemocytes toward foreign materials are easily observable *in vitro*.

In this paper, the classification of hemocytes, the identification of phagocytic cells in *Halocynthia roretzi* and their possible targets were studied *in vitro* by electron microscopy. The twofold aim of the present paper is to clarify the influence of plasma factors, pH and Ca^{2+}, Mg^{2+} ions in phagocytic activity for different targets.

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MATERIALS AND METHODS

Animals and hemocytes

Cultured solitary ascidians, *Halocynthia roretzi*, purchased from fisheries at Mutsu Bay, Asamushi, Aomori prefecture were used. The animals were maintained in laboratory aquaria containing natural sea water at 7–10°C without feeding and used for experiments within three weeks. Their morphological features did not change during that period. The hemolymph was collected into sterilized ice-cold tubes by cutting the tunic at the bottom of the body or from the space just beneath the tunic papilla at the upper part of the animal with a sterilized plastic syringe.

Preparations for electron microscopy

The hemocytes were suspended into 0.1% glutaraldehyde in 1.5% NaCl solution buffered with 0.2 M sodium cacodylate at pH 7.4. They were packed into a pellet by centrifugation (225×g, 5 min) 2 hr after fixation. The pellet was continuously fixed in the same fixative for 22 hr at room temperature and post-fixed in 1% osmium tetroxide in the same buffer for 2 hr on ice. Epoxy resin sections stained with uranyl acetate and lead citrate were observed with a JEM 100T electron microscope (Japan Electron Optics, Tokyo). The count of cell numbers with electron microscopy was performed on the sections

cut out from the different blocks taken from one fixed specimen.

Phagocytosis and encapsulation

The inorganic and organic targets used for phagocytosis or encapsulation were: Latex beads (LB) of 1.03 μm (small LB), 4.62 μm (middle LB) and 25.7 μm (large LB) in diameter as inorganic targets; fresh red blood cells of sheep (SRBC) (Nippon Bio-Test Inc.), SRBC fixed for 24 hr in 1% glutaraldehyde (gSRBC), *Escherichia coli* W3630 (a gift from Professor Masaya Kawakami, Kitasato University School of Medicine) and tunic pieces cut into 2 mm cubes (TP) as organic targets. The organic targets, except for the TPs, were washed three times in phosphate-buffered saline (PBS) and suspended in Pantin's artificial sea water (NaCl 23.51 g, $MgCl_2 \cdot 6H_2O$ 10.685 g, KCl 0.725 g, $CaCl_2$ 1.119 g, Na_2SO_4 3.937 g/l) [25] buffered with 20 mM HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid) at pH 7.2 (ASW). The freshly collected hemolymph and the washed hemocytes after being suspended in various media were incubated with these targets at 23 ± 1°C with agitation every minute. The hemocytes were fixed at 5, 10, 20 and 30 min of the incubation time for electron and light microscopic observations. The final concentrations of targets in incubation media were: LB, 0.05 w/v%; SRBC, 2.5×10^7 cells/ml; gSRBC, 2.9×10^7 cells/ml; *E. coli*, $1.3–1.6 \times 10^8$ cells/ml and TP, 5 pieces in 3 ml.

Effects of Ca^{2+}, Mg^{2+} and pH on phagocytosis of washed hemocytes

The pH of the hemolymph collected was immediately adjusted to 5.6 with one tenth volume of Ca^{2+}- and Mg^{2+}- free ASW containing 50 mM MES ([N-morpholino]ethanesulfonic acid) and 0.1 M EDTA for the prevention of hemocytes aggregation. Hemocytes were washed three times in Ca^{2+}- and Mg^{2+}- free acid ASW and resuspended in the following incubation media: ASW, Ca^{2+}- and Mg^{2+}-free neutral ASW containing 0.54 mM EDTA ($Ca^{2+} \cdot Mg^{2+}$ free ASW), ASW buffered with 20 mM MES at pH 5.6 (acid ASW) and Ca^{2+}- and Mg^{2+}- free acid ASW containing 0.54 mM EDTA ($Ca^{2+} \cdot Mg^{2+}$ free acid ASW). These media were used after sterilization by an autoclave. Each hemocyte suspension was incubated with small LB at 23 ± 1°C with agitation every minute. The final concentration of hemocytes and small LB were 2×10^7 cells/ml and 1×10^8 particles/ml, respectively. After dilution with ASW at 5, 10 and 20 min of the incubation, an aliquot of the mixture was poured on a slide glass and settled for 5 min in order for the hemocytes to adhere onto the glass surface and extend their pseudopodia. Specimens were poured off ASW, dried in air and stained with May-Grünward Giemsa solution. The phagocytic activity was shown as the ratio of the number of SG ingesting small LB to that of total SG counted under a light microscope.

The effect of hemolymph plasma on the phagocytic activity

After washing three times with $Ca^{2+} \cdot Mg^{2+}$ free acid ASW, the hemocytes (3×10^7 cells/ml) were incubated with SRBC or middle LB in their own plasma, plasma diluted with ASW or ASW. The concentration of the target particles was adjusted to the same as of the hemocytes. The phagocytic activities of SG in each medium for 20 min at 23 ± 1°C was measured with a light microscope in the same way as in the previous experiment.

RESULTS

Types and distribution of hemocytes

The mean density of cells in hemolymph from 5 animals was $2–3 \times 10^7$ cells per milliliter and did not markedly change during the 30 days' cultivation at 7–10°C in our aquaria. The twelve types of hemocytes, which were distinguished under an electron microscopic observation, were as follows: Small granular amebocytes (SGs), large granular amebocytes(LGs), dense granular cells, lymphoid cells and eight types of vacuolated or vesicular cells, V1-V8, which were classified by the size of the vacuole and the nature of inclusions (Fig. 1 and 2). Some intermediate and immature cells were occasionally observed besides the typical types.

Table 1 shows the composition of hemocytes which were measured in five animals from different batches reared in aquaria for 1–17 days. The percentages of SG, LG and V2 were not significantly different among the animals, that of other vacuolated cells, however, differed for each animal.

Small granular amebocyte (SG) (Fig. 1A) The nucleus of SG was irregular in shape and was usually in the center of the cell. Smooth tubulo-vesicular components of various sizes, some of which included electron dense material, and small granules about 0.2–0.4 μm in diameter were observed in the cytoplasm. The granules consisted of homogeneous and electron dense material, which was always more electron dense than LG, and some granules showed elongated shapes. The Golgi apparatus was at the peripheral region of the nucleus. The SG was obviously phagocytes, because several phagosomes, which varied in size and content, were scattered in the cytoplasm. Occasionally, the SG engulfed and degraded a hemocyte. These cells extended the pseudopodia in every direction and possessed high mobility on a slide glass. The SG was the only phagocytic hemocyte identified with a light microscope. The phagocytic processes of SRBC and middle LB by these cells were easily observed *in vitro*.

Large granular amebocyte (LG) (Fig. 1B) These cells were another type of granular amebocytes and their density was the second in line. The shape of the LG fixed immediately after collection was round but it changed to a rod shape about 8–13 μm in length with an irregular shaped nucleus *in vitro*. The Golgi apparatus and the rough endoplasmic reticulum were observed near the nucleus. Most of the cytoplasm was filled with spherical granules about 0.4–0.6 μm in diameter, larger than that of the SG and contained finely granular and homogeneous contents limited by a membrane. The LG elongated and extended its pseudopodia and some granules swelled *in vitro*. Bundles (50–125 nm in diameter) of short filaments (10–20 nm in diameter) appeared just beneath the cell surface when these cells were fixed by Karnovsky's fixative. They interlaced and were parallel to the membrane surface of both LG and SG.

Lymphoid cell (Fig. 1C) The lymphoid cells were about 3–5 μm in diameter and their cytoplasm had features similar to the lymphocytes of vertebrates.

Dense granular cell (Fig. 1D) The cells have a round shape about 4–5 μm in diameter. Extended types like SG and LG were not observed. The granules in the cytoplasm had a oval shape (about 0.4×0.6 μm) like the granules of the LG, and had almost the same electron density as the granules of

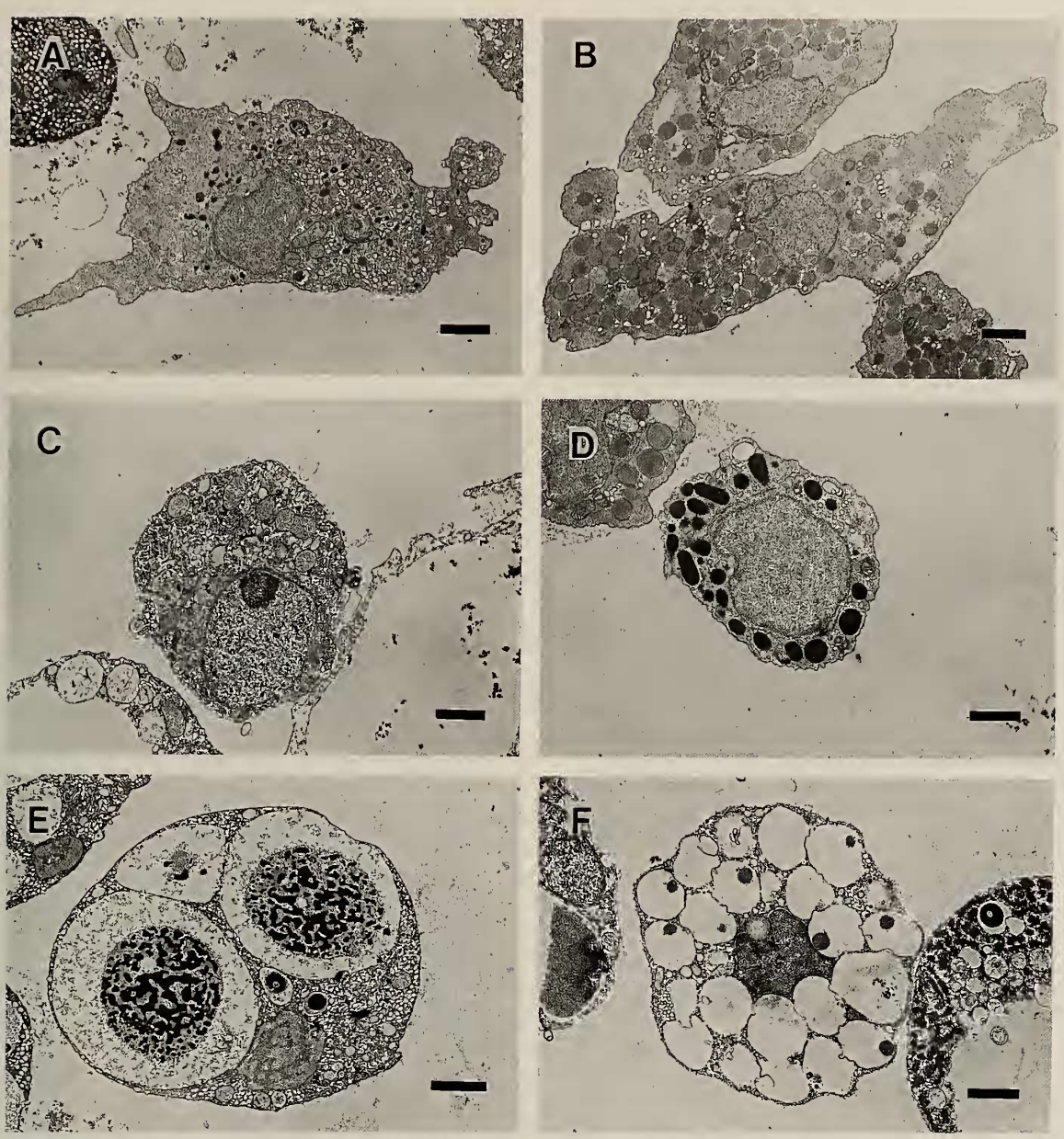

FIG. 1. The hemocyte types of *H. roretzi*. A: small granular amebocyte (SG), B: large granular amebocyte (LG), C: lymphoid cell (LY), D: dense granular cell (DG), E: V1, F: V2. Scale bars=1 μm.

the SG and were always more dense than the granules of LG.

Vacuolated or vesiculated cells The rest of the hemocytes were vacuolated or vesiculated cells. We distinguished eight types.

V1 (Fig. 1E) These cells were the most abundant cells in the hemolymph. They had a few large vacuoles, many small vesicles and a few small oval mitochondria in the cytoplasm, and an irregular shaped nucleus located at a peripheral side of the cytoplasm. It appeared that the vesicles fused each other to increase their sizes and their contents. They could finally become large vacuoles.

V2 (Fig. 1F) Many spherical vacuoles about 1–1.8 μm in size filled the cytoplasm with a nucleus at the central part of the cells. This type probably corresponds to a globular cell. Fine and dense granules were observed in the vacuoles.

V3 (Fig. 2A) Cells of this type had about ten vacuoles on one side of the cytoplasm in each section. Each vacuole contained electron dense and crescent-shape material.

V4 (Fig. 2B) These cells had many vacuoles and some of them contained fine granules or debris-like substance.

V5 (Fig. 2C) These cells were about 10–13 μm in diameter. The most characteristic feature of these cells was the existence of numerous vesicles about 0.2–0.6 μm in diameter and the well developed Golgi apparatus. The vesicles included very fine granules and the larger ones contain fibrous material. Their cytoplasm was stained basophilic by Wright or May-Grünward Giemsa stain.

V6 (Fig. 2D) Vacuoles with a homogeneous fine granular

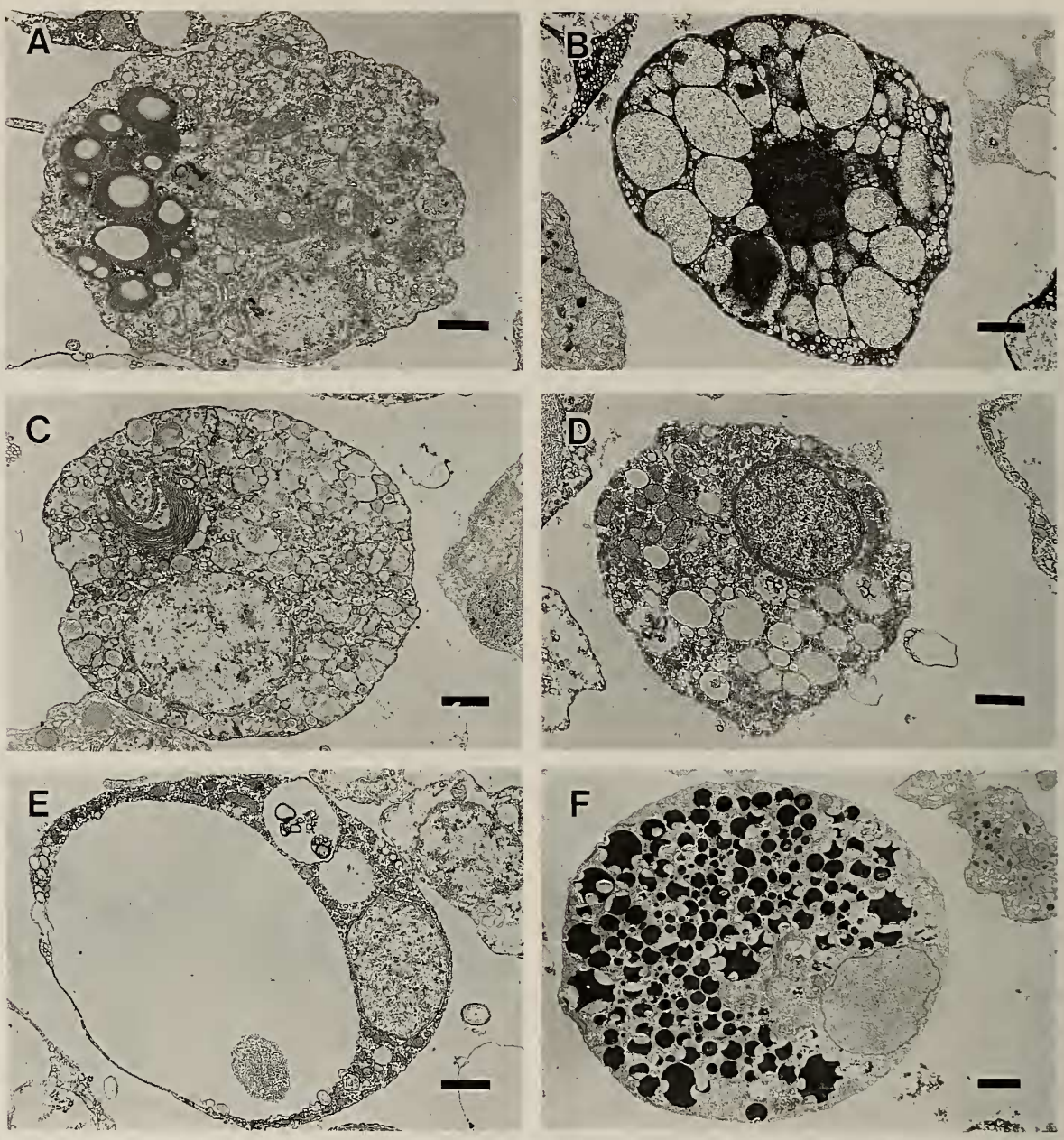

FIG. 2. The hemocyte types of *H. roretzi* (continued). A-F: V3-V8. Scale bars=1 μm.

content and vesicles in various sizes filled the cytoplasm. The nucleus was located peripherally in the cytoplasm.

V7 (Fig. 2E) This is a signet ring cell, very rare in the hemolymph of *H. roretzi*. These cells are about 6–12 μm in diameter and they have a single large vacuole occasionally containing granular material. The nucleus and their cytoplasm were located in the periphery of the cell.

V8 (Fig. 2F) Cells of this type were filled with a lot of very electron dense vacuoles and had an eccentric and crescent shape which could be an artificial result from the fixative used. The nucleus of these cells was located at a peripheral side of the cytoplasm.

Electron and light microscopic observations on phagocytosis and encapsulation to some inorganic and organic targets

When the hemolymph was mixed with small LB *in vitro*, many SGs were actively phagocytosed (Fig. 3). But only a part of the LGs could ingest a few small LBs (Fig. 4). It was observed in a sample taken one minute after mixing that a SG was extending its pseudopods to encircle a particle and had already ingested particles in the phagocytic vacuoles in its cytoplasm. In Fig. 3 eleven particles can be seen in the cytoplasm of one SG fixed 10 min after the onset of incubation.

The phagocytic activity to small LB by LG was less than that by the SG. Though many particles were observed among LGs in an aggregate induced in the hemolymph after

TABLE 1. The hemocyte composition in the hemolymph of *H. roretzi*

Cell types	Composition (%)
Small granular amebocyte	29.8±1.6
Large granular amebocyte	10.7±4.8
Dense granular cell	0.4±0.2
Lymphoid cell	0.4±0.1
Vacuolated or vesicular cell	
V1	42.2±6.0
V2	5.4±2.6
V3	3.1±0.8
V4	3.0±1.4
V5	2.4±0.9
V6	2.1±0.9
V7	0.4±0.1
V8	0.2±0.1

Percentage values represent the mean±SD of five animals from different batches kept in an aquarium for 1–17 days.

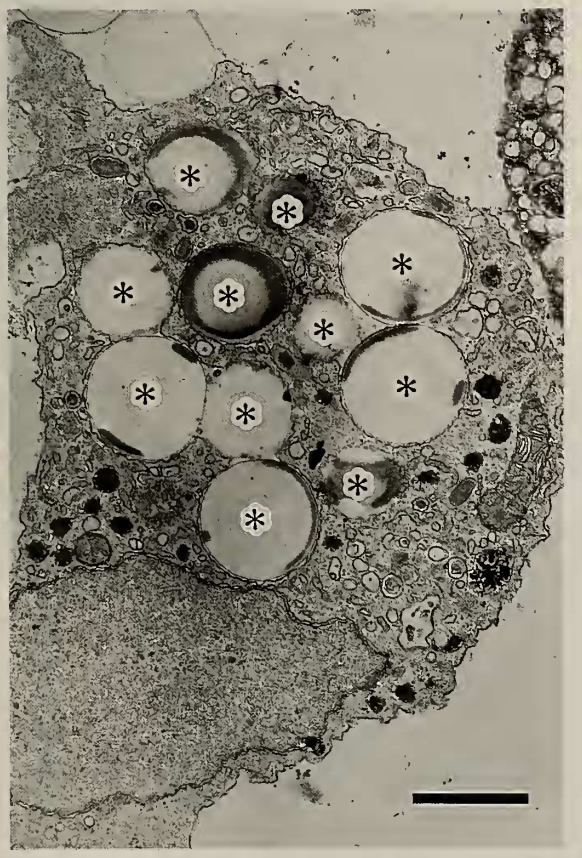

FIG. 3. A SG ingesting small LB (*). Eleven LBs are seen in the cytoplasm of a SG 10 min after the incubation. Scale bar=1 μm.

the addition of small LBs (Fig. 4). The numbers of SGs and LGs, which phagocytosed small LBs in the hemolymph *in vitro*, increased with the progress in the incubation time (Table 2). Twenty percent of SGs have already ingested the

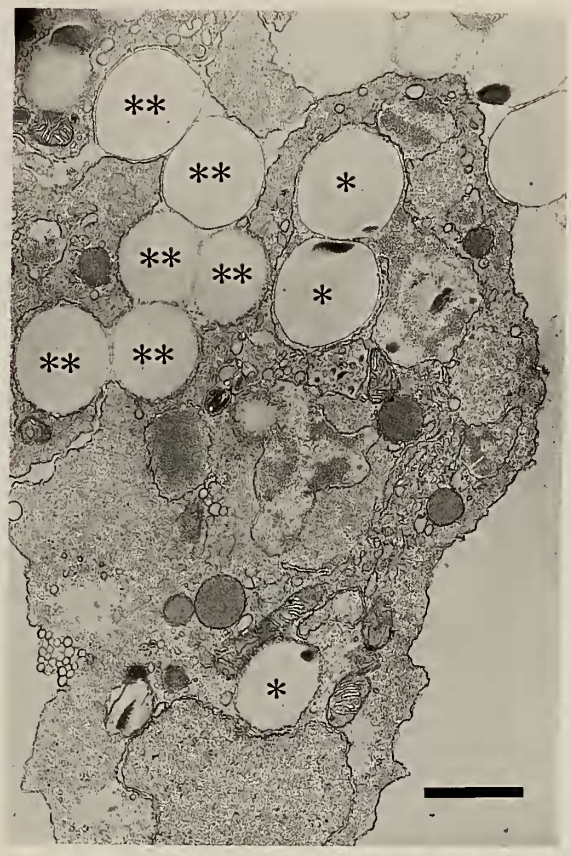

FIG. 4. A LG ingesting 3 small LBs 10 min after the mixing. *: Small LB in the cytoplasm. **: Small LB outside of cells. Scale bar=1 μm.

TABLE 2. The time-dependent increase of the numbers of SG and LG phagocytosing small LB

Hemocytes	Number of cells ingesting small LB			
	1 min	5 min	10 min	30 min
SG	254/1283 (19.8%)	711/1433 (49.6%)	990/1346 (73.6%)	1153/1293 (89.2%)
LG	0/411 (0%)	16/350 (5.0%)	23/413 (5.6%)	27/290 (9.3%)
Others*	0	0	0	0

Values express the number of phagocytosing SGs (LGs)/total SGs (LGs) in the sum of results obtained from three different animals. Parcentages are shown in parenthesis. *: Hemocytes more than 3000 were counted in every observations.

particles within one minute of the incubation and 89% of SGs ingested the particles for 30 min. Only 9.3% of LGs phagocytosed the particles even 30 min after the onset of incubation, and the number of LBs ingested in the cells was less than that of SGs. We could not find any phagocytosed particle in the cytoplasm of other hemocyte types though we counted more than 3000 cells under electron microscopic observations.

When middle LBs were applied to the hemolymph as targets almost the same size as SGs, only the SGs were able to

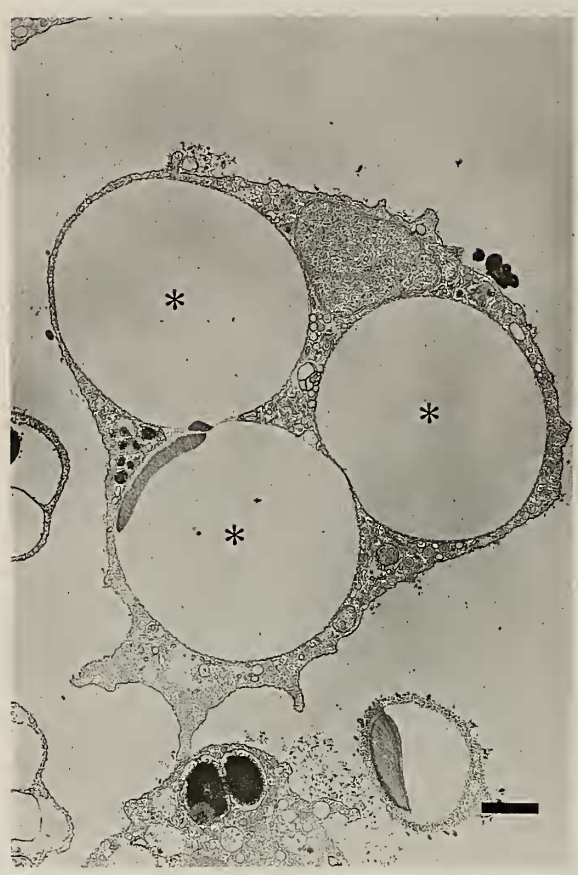

FIG. 5. A SG ingesting 3 middle LBs (*) in the cytoplasm after the 30 min incubation. Scale bar=1 μm.

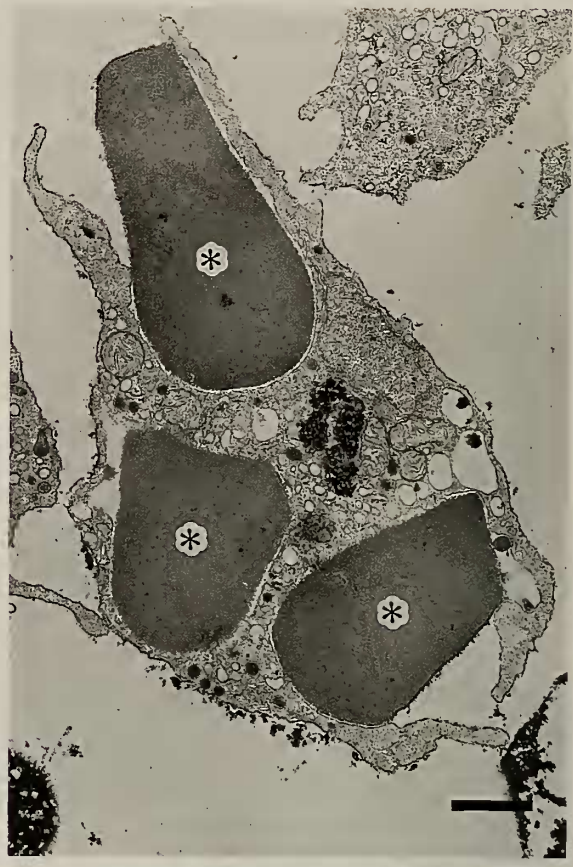

FIG. 7. A SG ingesting glutaraldehyde fixed SRBC (*) 5 min after mixing. gSRBCs which were caught by pseudopodia (upper), already ingested in the cytoplasm (middle) and just included (lower) are shown. Scale bar=1 μm.

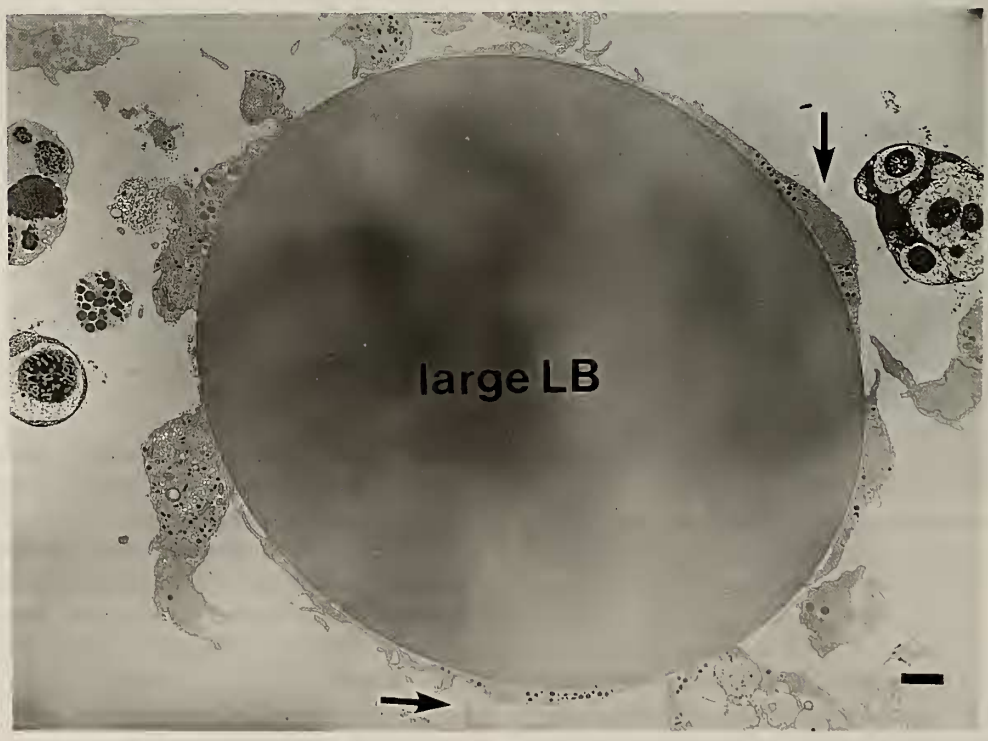

FIG. 6. SGs adhering to the surface of a large LB after the 30 min incubation. Arrows indicate SGs. Scale bar=2 μm.

ingest particles within the 30 min incubation (Fig. 5). No middle LB was observed in hemocytes of other types even 30 min after the onset of incubation.

When large LBs were added into the hemolymph, SGs adhered on the surface of large LBs to encapsulate them by elongation and the spreading of their pseudopodia (Fig. 6).

The organic particles, SRBC, gSRBC or *E. coli*, were also phagocytosed only by SGs in the hemolymph. Figure 7 shows gSRBCs which have been ingested in a SG, just included and caught by pseudopodia in a sample fixed five min after mixing. In this sample, the surface of the pseudopodia does not always make contact with the surface of the targets. *E. coli* was also well ingested by SGs but not by LGs although some of the LGs were able to phagocytose small LBs which have almost the same size as *E. coli* (about 1 μm in diameter) (Fig. 8).

When the hemolymph was incubated with pieces of the tunic dissected from the same animals, SGs tightly spread over a tunic piece and became a single layer surrounding it 30 min later (Fig. 9).

The comparison of phagocytic activity of hemocytes toward different targets

The phagocytic ability of SG and LG in the hemolymph *in vitro* toward different foreign particles was compared in 30 minute-incubation of hemolymph using small, middle and large LB as inorganic targets and SRBC, gSRBC and *E. coli* as organic targets (Table 3). The phagocytic reaction of the SGs to the inorganic targets was more intense than to the

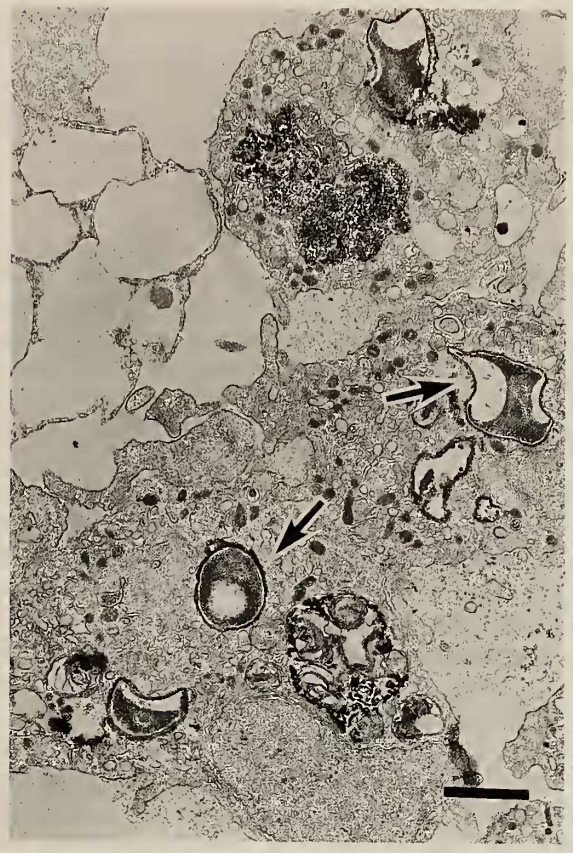

FIG. 8. A SG ingesting *E. coli* (arrows) 10 min after mixing. Scale bar=1 μm.

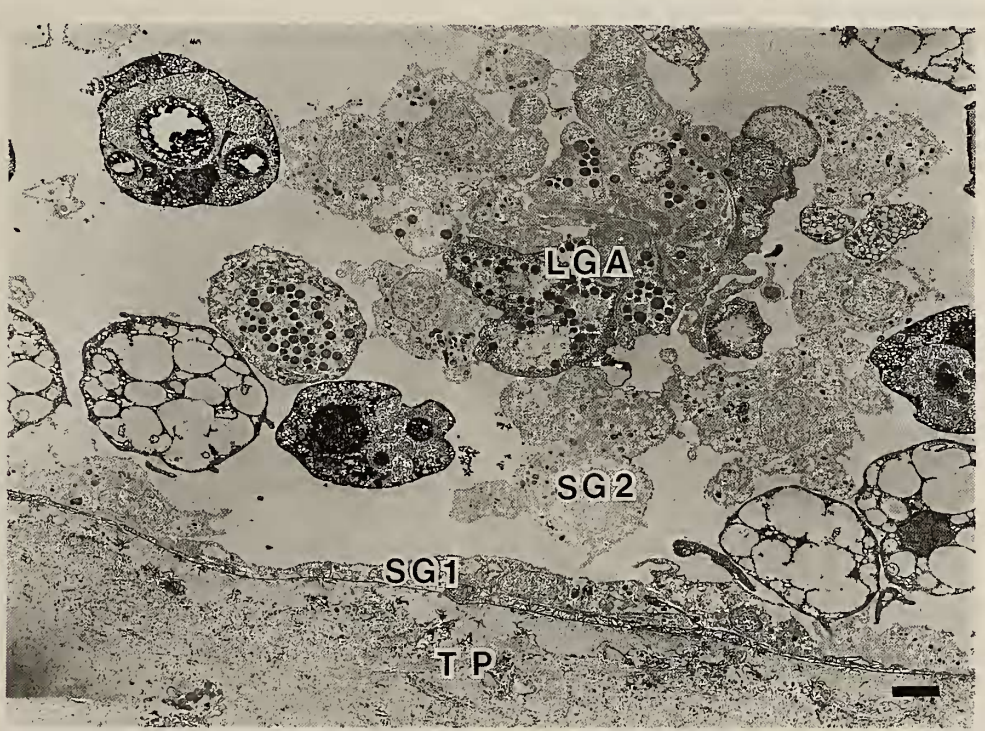

FIG. 9. SGs spreading over the surface of a tunic piece and surrounding an aggregate of LGs 30 min after incubation. SG1: The SG covering the surface of a tunic piece, SG2: The SG surrounding an aggregate of LGs, LGA: An aggregate of LG, TP: A tunic piece incubated with the hemolymph. Scale bar=2 μm.

TABLE 3. The phagocytic activity of hemocytes toward different targets

Targets	% of cell ingesting targets		
	SG	LG*	Others**
Latex beads (ϕ 1.03 μm)	89.3±0.3	10.1±1.47	0
(ϕ 4.62 μm)	83.2±2.54	0	0
SRBC	74.8±2.32	0	0
gSRBC	78.2±5.0	0	0
E. coli	79.9±3.25	0	0

Values represent the mean±SD of experiments from three animals as percentages of cells ingesting the particle of the total number of each cell type. Hemocytes were incubated with particles for 30 min. *: LGs more than 300 were counted in every experiment. **: Hemocytes more than 3000 were counted in every experiment.

organic ones and that to small LB was the highest. The reaction to the gSRBC was stronger than to the SRBC and almost the same as that to *E. coli*. On the other hand, the LGs showed low phagocytic activity only toward small LB.

The effect of Ca^{2+} and Mg^{2+} ions, and low pH on the phagocytic activity of SG

The effect of Ca^{2+} and Mg^{2+}, and pH on the phagocytic activity of SG to small LB was tested using washed hemocytes. The phagocytic activity was measured under light microscopy after a May-Grünward Giemsa stain. The results are summarized in Table 4. SG was easily identified by the following characteristics in the light microscopic preparates: Adhering to a slide glass with elongated pseudopodia and containing acidophilic fine granules in the cytoplasm. In the hemolymph, 53% of SGs phagocytosed the particles for five min and 77% for 20 min of the incubation time. When the hemocytes were washed and resuspended in ASW, the phagocytic activity of the SG became stronger than in the hemolymph. Further, when the washed hemocytes were suspended in acid ASW and/or Ca^{2+}·Mg^{2+} free ASW, more than 80% of SGs have phagocytosed the small LB within five min and 94% of the cells within 20 min of incubation. The phagocytic activity in these media was significantly higher than in the hemolymph.

TABLE 4. The effect of the incubation media on the phagocytic activity of SGs against small LB

Incubation media	% of SG ingesting small LB		
	Incubation time		
	5 min	10 min	20 min
Normal hemolymph	52.9±6.39	61.2±4.41	77.0±3.73
ASW	73.6±6.68*	82.2±5.41*	86.6±4.72*
Ca^{2+}·Mg^{2+} free ASW	84.1±3.16**	92.9±3.36**	93.9±3.42**
Acid ASW	83.9±3.84**	91.8±3.97**	93.7±2.51**
Ca^{2+}·Mg^{2+} free acid ASW	88.3±4.12**	90.6±4.07**	93.8±2.27**

Values represent the mean±SD of experiments from five animals as percentage of SGs ingesting small LB of a total SGs. *: The values of ASW were significantly higher than those of normal hemolymph ($P<0.01$). **: The values of Ca^{2+}·Mg^{2+} free ASW, Acid ASW and Ca^{2+}·Mg^{2+} free acid ASW were higher than those of normal hemolymph and normal ASW ($P<0.01$).

The effect of hemolymph plasma on phagocytosis of SGs

Hemocytes and hemolymph plasma were obtained from the same animal. The washed hemocytes were resuspended in the hemolymph plasma, the diluted plasma or ASW and incubated with SRBC or middle LB for 20 min. The concentration of target particles was adjusted so it was the same as the hemocyte concentration in each experiment (3×10^7 cells/ml). The results are shown in Table 5. SGs actively phagocytosed the middle LB without any significant difference between those in ASW and those in plasma. On the other hand, the phagocytic activity of SG to SRBC was significantly influenced by the concentration of plasma in the incubation mixture. Within 20 min after the start of incubation 71% of SGs phagocytosed the SRBC in the hemolymph, but only 39% of SGs phagocytosed the SRBC in ASW. The higher the plasma concentration was in the incubation medium, the more the active SGs increased.

TABLE 5. The effect of plasma on the phagocytic activity of SGs

Media	% of SG ingesting targets	
	SRBC	middle LB
ASW	39.1±1.86[a]	69.2±3.08[f]
ASW:Plasma=9:1	41.6±4.93[b]	—
ASW:Plasma=1:1	56.5±5.24[c]	—
Plasma	67.0±1.97[d]	—
Hemolymph	71.0±4.55[e]	69.9±2.99[g]

Values represent the mean±SD of experiments from five animals. The plasma used was obtained from the same individual as for the cell sourse. Hemocytes were incubated with particles for 20 min. Significant differences are recognized between a and c; b and c; and c and d ($P<0.01$) but not between a and b; d and e; and f and g.

LG aggregation induced by the addition of foreign particle and TP

The addition of foreign particles into the hemolymph induced the aggregation of LGs, even if they were TPs from the same animal. The granules of LGs in the center of the aggregate swelled and fused each other (Fig. 4, 9). It was also observed that SGs were surrounding the aggregate of LGs (Fig. 9) in the same manner as with large LB and TP.

DISCUSSION

There are many reports which propose and demonstrate the relationship of the structure of ascidian hemocytes to various functions such as nutrition [11], tunic formation [9,

10], heavy metal accumulation [9, 21], germ cell formation [11] and immune responses [1, 19, 34]. A wide variety of cell types found among different species and their various functions are well known. Overton [24], using *Perophora viridis*, has reported with a special reference to the fine structure of vanadocyte. Milanesi and Burighel [22] and Burighel *et al.* [7] distinguished six cell types based on the fine structure in *Botryllus schlosseri*. It is generally believed that six to nine cell types of hemocytes are found in the hemolymph of ascidians [35], however, the relationship between the physiological roles and morphological characters is not clear.

Recently, Sawada *et al.* [28] classified ten groups of living hemocytes of *H. roretzi* (two types of phagocytes, three types of granular cells, four types of vacuolated cells and lymphoid cells) according to vital staining, autonomous fluorescence and the effect of NH_4 ions in the presence of EGTA at pH 6.0, where hemocytes were adhering or spreading on glass. Zhang *et al.* [37] identified nine types of hemocytes in *H. roretzi* according to their ultrastructural characteristics. Fuke and Fukumoto [15] classified hemocytes of *H. roretzi* into nine types by electron microscopy with reference to their appearance by light microscopy (vacuolated cells, hyaline amoebocytes, small amoebocytes, granular amoebocytes, macrogranular cells, globular cells, lymphocyte-like cells, large basophilic cells, and large granular cells).

Referring to the above results, we classified hemocytes of *H. roretzi* into twelve types on ultrastructural findings as follows: SG, LG, lymphoid cells, dense granular cells and eight types of vacuolated or vesicular cells, V1-V8, which were distinguished by the size of vacuoles and the nature of the inclusion. Table 6 shows our classification as we believe it corresponds to Sawada *et al.* [28] and Fuke and Fukumoto [15]. The dense granular cells, V3, V4, V6 and V7 that we observed are not described by them.

Ascidians have got two defense mechanisms against foreign materials (bacteria, certain species of copepoda, non-self tissues). The first, known as humoral responses, involves natural agglutinins [3, 12, 32, 34, 36] and bactericidines [2, 20]. We also found in the hemolymph plasma of *H. roretzi*, the existence of protease and its inhibitor, which are activated by contact with the tunic *in vitro* (Unpublished data). The second is cellular responses that include inflammatory-like reactions, phagocytosis, encapsulation and histocompatibility [1, 26, 31, 35].

Phagocytic activities of several ascidians' hemocytes were reported under experimental conditions. Small particulate foreign bodies were introduced into the tunic or vascular system, and were phagocytosed by hyaline and granular leucocytes [35]. Carmine particles were phagocytosed readily in the cytoplasm of amebocytes and trypan blue could be identified in cytoplasm of amebocytes and vacuoles of signet ring cells after intracardial injections into *Molgula manhattensis* [1]. Fuke [13] reported phagocytic activity in *H. roretzi* of the following cells: Vesicular cells, fine granular ameboid cells, minute granular cell, small vacuolated cells and large basophilic cells. On the other hand Sawada *et al.* [28] confirmed phagocytic activity toward SRBC in three groups of hemocytes of *H. roretzi*. But they supposed that the pahgocytes of this species consist of two types of amebocyte (p-1, p-2). Although many types of the hemocytes showed certain signs of endocytosis under electron microscopic studies in other ascidians, only one type of cells is called the phagocyte or the macrophage because these cells frequently contain one or more cells in their cytoplasm [22, 24]. In our present electron and light microscopic observations, however, only SGs showed active pahgocytic activity toward not only inorganic targets like LB but organic targets

Table 6. The names and symbols provided by us for each cell type and the corresponding names by Sawada *et al.* and by Fuke & Fukumoto

Cell types	Corresponding names	
	Sawada *et al.** LM	Fuke & Fukumoto** TEM
Small granular amebocyte	p1 and p2	Hyaline amoebocyte and Small amoebocyte
Large granular amebocyte	g1	Granular amoebocyte
Dense granular cell		
Lymphoid cell	Lymphoid cell	Lymphocyte-like cell
Vacuolated or vesicular cell		
V1	v3	Vacuolated cell T2
V2		Globular cell
V3		
V4		
V5	g2	Large basophilic cell
V6		
V7		
V8	g3	Large granular cell

*: According to Sawada *et al.* [28]. **: According to Fuke & Fukumoto [15].

like SRBC as well. We suppose that the clearing and elimination of foreign substances, such as bacteria, are mainly performed by the phagocytosis of SGs. The meaning of the phagocytosis carried out by a small number of LGs toward small LB only is not clear. LGs may have pahgocytic subpopulation(s) or they may exert their phagocytic activity in free cell conditions but lose it after their aggregation. The aggregation of LGs *in vitro* was induced within five min by the addition of small pieces of tunic or other foreign materials used in this experiment and the adhesion of SGs around LG-aggregate was also observed (details of the aggregate formation are not shown).

The encapsulation responding toward copepods have been naturally observed in the outer wall of the peribranchial cavity or within the gill cavity. The parasites were surrounded by a thin membrane of host tissue [6, 23]. The process of encapsulation has been studied in *M. manhattensis* in which hemocytes responsible for encapsulation reactions are vanadocytes and signet ring cells. These cells coat the glass fragment inserted in the branchial tissue [1]. In general, hemocytes responsible for the encapsulation reactions belong to those of the vacuolated category, predominantly the morula cell [35]. In the present observations, however, SG was the only responsible hemocyte for the encapsulation reaction of this species. It is not clear whether the differences of cell types responding for encapsulation reflected the differences of species, of cell appearance or of unique experimental conditions.

SGs readily ingested LBs in every condition of experimental media. As LBs used in this experiment protrude $-COOH^-$ group on their surface, they may easily have contact with the plasma membrane of SGs. In mammalian macrophages, anionic molecules are better inducers of phagocytosis than either neutral or cationic species [8]. The phagocytic activity of SGs toward gSRBC was higher than that of non-treated SRBC (Table 3). Usually, the outer surface of protein molecules exposes hydrophilic molecules. After denaturation, like in a treatment with glutaraldehyde, the hydrophobic groups are exposed to aqueous solvent. The increase in surface hydrophobicity could act as a marker, which would allow non specific recognition of macrophage [27, 33].

The highest phagocytic activity of SGs toward small LB was observed in Ca^{2+}·Mg^{2+} free acid ASW (Table 4). Because the aggregation of LGs and the adhesion of SGs to LG-aggregates were depressed in these condition, the chance of SGs striking their targets may have been increased.

Immunoglobulins are not known in the hemolymph plasma in ascidians. However, it has been suggested that ascidians have lectins that act as opsonin for foreign materials [3, 32]. The present experiment clearly demonstrates that humoral factor(s) in the hemolymph plasma play a significant role in the phagocytosis of SGs toward SRBC, but not toward middle LB.

Our results suggest that SGs play a part in the clearing and elimination of foreign materials together with a certain plasma factor and SGs and LGs play important roles in hemostasis and wound healing in *H. roretzi*.

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